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Reactivity of Antibodies from Syphilis Patients to a Protein Array Representing the *Treponema pallidum* Proteome†

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To identify antigens important in the human immune response to syphilis, the serum antibody reactivity of syphilitic patients was examined with 908 of the 1,039 proteins in the proteome of *Treponema pallidum* subsp. *pallidum* using a protein array enzyme-linked immunosorbent assay. Thirty-four proteins exhibited significant reactivity when assayed with human sera from patients in the early latent stage of syphilis. A subset of antigens identified were further scrutinized for antibody reactivity at primary, secondary, and latent disease stages, and the results demonstrate that the humoral immune response to individual *T. pallidum* proteins develops at different rates during the time course of infection.

Over the past half century, effective antibiotic treatment programs have made syphilis relatively uncommon in the United States, with less than 7,100 primary and secondary cases diagnosed in 2003 (6). However, recent data indicate that reported cases are again increasing in subsets of the population, and periodic epidemics of syphilis have occurred for decades (5). In 1995, the number of new cases of syphilis worldwide was estimated to be 12 million per year (29).

As a syphilitic infection can produce a variable range of symptoms in humans, laboratory tests are often required to definitively diagnose an infection. Due to the inability to culture the organism in vitro, a need exists for the development and optimization of *T. pallidum* detection in diverse clinical specimens (16). While enzyme-linked immunosorbent assays (ELISAs) for *Treponema* are commercially available, they exhibit varying efficiencies at different disease stages (23). Thus, knowledge of the presence and timing of antigenic protein expression by *T. pallidum* will allow for the selection of optimal antigen combinations for *T. pallidum* detection.

New cases of syphilis occur primarily in areas of poor health care and low socioeconomic status (19), and the availability of a vaccine would greatly aid in reducing the worldwide incidence of the disease. A syphilis vaccine could reduce dependence on antibiotics, prevent side effects due to antibiotic administration, and prevent disease before it occurs. Indeed, the Centers for Disease Control and Prevention has included the development of a vaccine in their plan to eradicate syphilis from the United States (13). In 1973, complete protection from reinfection with *Treponema pallidum* subsp. *pallidum* was seen in rabbits immunized with large numbers of gamma-irradiated treponemes (15). However, the large numbers of organisms and injections used render this approach impractical for human vaccine development. Several syphilitic manifestations can be reproduced in the rabbit, and experimental infection of rabbits has been shown to be an effective vehicle to test vaccine candidates (4). Optimally a subunit vaccine of recombinantly expressed proteins or peptides could be developed. Although many vaccine candidates have been tested, thus far no antigen has been shown to provide complete protection from subsequent *T. pallidum* infection.

Previously, we performed a systematic screen of the *T. pallidum* proteome to identify antigenic proteins during rabbit infections (13). In order to identify novel human antigens, we have extended this study to a large-scale screening of the *T. pallidum* proteome using sera collected from patients with syphilis. Our results reveal many newly identified antigens that can be further characterized for vaccine potential as well as for clinical diagnostic purposes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21(DE3) (Invitrogen, Carlsbad, Calif.). Plasmids expressing GST-*T. pallidum* subsp. *pallidum* strain Nichols fusion proteins were constructed using PCR amplification of each *T. pallidum* gene, ligation into a donor plasmid, and Cre-*loxP* recombination with a GST expression vector using Invitrogen's Univector cloning technology as previously described by McKevitt et al. (14). *E. coli* cells were cultured in Luria Bertani (LB) or 2YT medium (16% [wt/vol] Bacto-Tryptone, 1% [wt/vol] Bacto-Yeast, 0.5% [wt/vol] NaCl).

Serum preparation. The human serum samples were previously collected in Texas from normal human subjects and from patients diagnosed with primary, secondary, and early latent syphilis. Sera were pooled prior to the ELISA experiments as normal human sera (six sera), primary (two sera), secondary (nine sera), and early latent (five sera). For the initial screening of reactivity, the pool of sera from five patients with early latent syphilis was used. Human sera collected from patients diagnosed with secondary syphilis were kindly provided by Robert Baughn, VA Medical Center, Houston, TX. All human sera were collected under established guidelines with prior approval by the Committee for the Protection of Human Subjects, University of Texas Health Science Center at Houston.

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Absorption of anti-*E. coli* protein antibodies. Before use, serum samples were incubated with *E. coli* cell lysate to remove nonspecific reactivity. Briefly, *E. coli* BL21(DE3) was grown overnight at 37°C in LB medium. Cell pellets were resuspended in 10 ml bacterial protein extraction reagent (B-PER) (Pierce, Rockford, Ill.) containing 0.375 mg/ml lyzozyme and 420 ng/ml DNaseI and then incubated on a rocking platform for 10 min at room temperature. Cell debris was deposited by centrifugation (10 min at 16,325 × *g* at 4°C), and the supernatant was collected for use. A mixture of 10 μl serum, 11 ml phosphate-buffered saline (PBS) (pH 7.4) containing 1% dry milk, and 1 ml BL21(DE3) cell lysate supernatant was mixed on a rocking platform for 2 h at room temperature just prior to use.

Protein expression. Expression conditions were optimized and standardized as previously described (13, 14). Briefly, *E. coli* BL21(DE3) hosting the plasmid constructs containing individual *T. pallidum* open reading frames (ORFs) was inoculated into 1 ml LB media containing 25 μg/ml kanamycin, 100 μg/ml ampicillin, and 2% glucose in 96-well plates. Following incubation with shaking overnight at 37°C, 100 μl of the culture was added to 1.5 ml 2YT containing 25 μg/ml kanamycin and 100 μg/ml ampicillin. The cultures were incubated in 2-ml wells containing microstir bars in a 96-well format at 30°C for 5 h, followed by addition of isopropyl-ã-D-thiogalactopyranoside (0.1 mM final concentration) and incubation for an additional 5 h. Cells were then pelleted and stored at −80°C. Previous control experiments indicated that the *T. pallidum* GST fusion proteins vary in the amount of protein expression in *E. coli* based on reactivity of an anti-GST antibody but that there was not a strong correlation between the amount of anti-GST reactivity and the amount of reactivity with sera from rabbit infections (14).

ELISA protocol. Each pellet was subjected to three rounds of freeze-thawing prior to addition of 220 μl of bacterial protein extraction reagent (B-PER) (Pierce, Rockford, Illinois) containing 0.375 mg/ml lysozyme and 420 ng/ml DNaseI, which was used to lyse the pellet. The resuspended pellets were stirred vigorously with a microstir bar at room temperature for 10 min. Reacti-Bind glutathione-coated white 96-well plates (Pierce, Rockford, Ill.) were blocked overnight in 150 μl PBS-Casein (Pierce, Rockford, Ill.). A volume of 110 μl of each cell lysate was added to the glutathione-coated plates, followed by incubation at room temperature for 2 h. The plates were washed with 210 μl of PBS (pH 7.4)–0.05% Tween 20 (buffer A) using an Elx50 Auto Strip washer (Bio-Tek, Winooski, Vt.) eight times, and the wells were then blocked with 150 μl PBS (pH 7.4) containing 5% dry milk at room temperature for 1 h. Absorbed human serum was diluted into buffer A to a final serum dilution of 1:1,200. A volume of 110 μl of the serum preparation was added to each well, followed by incubation for 2 h at room temperature. The plates were then washed eight times with buffer A. A 1:12,000 dilution of goat anti-human immunoglobulin G (IgG) and IgM horseradish peroxidase conjugate (110 μl; heavy plus light chain specific and affinity purified [Pierce, Rockford, Ill.]) was added to each plate well and was incubated for 1 h at room temperature. In a separate experiment under identical ELISA conditions, the goat anti-human antibody was shown to detect as few as 5 ng/well of purified human IgG (Pierce, Rockford, Ill.) and IgM (Pierce, Rockford, Ill.). The plates were washed eight times with buffer A before the addition of 150 μl of SuperSignal ELISA Pico chemiluminescent substrate (Pierce, Rockford, Ill.). Light emission from each plate well was monitored 10 min after peroxidase substrate addition with a Genios plate reader (Tecan, Durham, N.C.) for 200 ms. The experiments were repeated three times.

Data analysis. Each plate in the serum arrays contained immobilized GST without a fusion protein as a negative control in order to identify statistically relevant reactive proteins arrayed on the same plate. For analysis of the data generated from the arrays with human serum, the ratio between the chemiluminescence detected from a sample well containing a *T. pallidum* protein fused to GST and the chemiluminescence detected from a sample well containing only immobilized GST protein was calculated. These experiments were conducted three times, and the sample signal-to-background-signal ratios were averaged. Based on the addition of the mean of the reactivity of the normal human sera control plus four times the standard deviation (99.9% confidence interval assuming a normal distribution), a value of 1.5 or greater indicates significant interactions between antibodies present in the syphilitic sera and immobilized *T. pallidum* protein.

RESULTS AND DISCUSSION

Identification of antigenic proteins. Of the 908 *T. pallidum* proteins examined for reactivity with pooled early latent human serum, 34 proteins were considered significantly antigenic as indicated by a signal-to-background ratio of 1.5 or greater (Fig. 1; Table S1 in the supplemental material). The 34 proteins reactive with early latent human sera were also found to be significantly reactive in a previous study that used sera from *T. pallidum*-infected rabbits in a similar assay (13). This correlation validates our previous results as well as the use of the rabbit model system for antigen identification. A total of 90 proteins, 32 of those reactive with early latent human sera as well as additional proteins known to be antigenic in previous studies with rabbit sera, were selected for further analysis of reactivity with human sera from different stages of disease progression, including sera from patients with primary syphilis, secondary syphilis, or normal human sera (Table S2 in the supplemental material). Thirty-eight of the 90 proteins examined exhibited signal-to-background ratios of >1.5 with sera at one or more of the syphilitic stages (Table 1). Fourteen were reactive with the pooled sera from each stage and thus may represent good candidates for immunodiagnostic assays. Sixteen of the 38 antigens we identified were previously reported in the *T. pallidum* literature as antigens (Table 1), and only two proteins, TP0974 (hypothetical protein) and TP1015 (N utilization substance protein B), did not produce a detectable reaction in our previous immunoproteome analysis using sera from *T. pallidum*-infected rabbits (13).

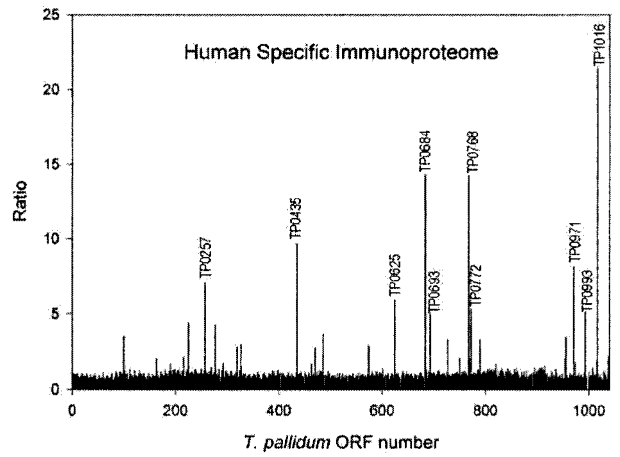

FIG. 1. Identification of antigenic proteins in the *T. pallidum* proteome using sera collected from patients in the early latent stage of disease. The chemiluminescence ratio refers to the relative light units resulting from the binding of serum Ig to the *T. pallidum*-GST fusion protein divided by the value obtained when wells were coated with GST alone.

As seen in Table 1, no significant interactions were detected when normal human serum was incubated with the arrayed proteins, and the most reactive syphilitic disease stage was the early latent stage. Six of the 38 reactive proteins, TP0133 (hypothetical protein), TP0136 (hypothetical protein), TP0326 (outer membrane protein), TP0398 (flagellar hook-basal body complex protein), TP0663 (outer membrane protein, putative), and TP0767 (translation elongation factor G), did not exhibit reactivity with early latent syphilis sera (Table 1). An immune response to these proteins may be specific to the early stage of infection, thus making them good candidates for a diagnostic test for early syphilitic infection. In our assay, 11 proteins were reactive (ratio, ≥1.5) with the early latent pool, but they were not reactive with sera from primary or secondary syphilis pa-

TABLE 1. 38 *T. pallidum* proteins that exhibit signal-to-background ratios of >1.5 with sera from one or more syphilitic disease stages[a]

ORF no.	Name and/or function	Source (s) of previously identified antigens	Signal sequence prediction[b]	Predicted molecular weight	pI	Normal ratio, human sera	Primary ratio, human sera	Secondary ratio, human sera	Early latent ratio, human sera
100	Thioredoxin, putative	26	I	21,994	8.88	0.9	**4.0**	**1.8**	**3.6**
133	Hypothetical protein		TMH	43,215	6.71	1.2	**2.4**	1.2	1.1
136	Hypothetical protein		TMH	50,145	7.68	1.2	**2.1**	1.0	0.7
163	ABC transporter, periplasmic binding protein	2	II	33,557	6.68	0.8	0.9	0.8	**2.0**
216	Heat shock protein 70	17	CYT	68,033	4.89	0.8	0.8	0.9	**2.2**
225	Leucine-rich repeat protein TpLRR	25	I	26,555	9.09	1.1	**2.1**	**1.6**	**4.4**
257	Glycerophosphoryldiester phosphodiesterase	27	II	41,002	10.04	1.0	**7.3**	**3.0**	**7.1**
277	Carboxyl-terminal protease		CYT	50,306	9.97	0.9	0.9	0.9	**4.3**
292	Conserved hypothetical protein	10	CYT	47,597	6.88	1.0	1.2	1.0	**1.7**
319	Membrane lipoprotein	24	II	37,757	4.61	0.8	**6.2**	**3.7**	**2.8**
326	Outer membrane protein	4	I	96,123	9.29	0.9	**2.6**	1.2	1.49
327	Cationic outer membrane protein		I	19,700	8.99	0.8	1.0	0.9	**3.0**
398	Flagellar hook-basal body complex protein (FliE)			13,687	7.53	0.9	**1.6**	1.0	1.2
435	Lipoprotein, 17 kDa	21	II	16,451	8.7	1.2	**16.1**	**16.6**	**9.6**
463	Hypothetical protein		CYT	11,812	4.61	0.8	**6.1**	**1.7**	**1.7**
470	Conserved hypothetical protein		I	43,291	9	0.7	1.1	0.8	**2.8**
486	Antigen, p83/100		CYT	53,365	9.19	0.7	**2.4**	**1.9**	**3.6**
574	Carboxypeptidase, 47 kDa	12	II	47,654	5.41	0.7	**10.0**	**3.7**	**2.9**
625	Hypothetical protein		CYT	28,860	9.68	0.9	**1.7**	1.3	**5.9**
663	Outer membrane protein, putative (Tromp 2)	8, 18		27,132	7.41	0.8	**1.9**	1.0	0.8
684	Methylgalactoside ABC transporter, periplasmic galactose-binding protein	1	II	43,041	5.05	0.7	**19.0**	**6.8**	**14.3**
693	Hypothetical protein		II	47,661	6.81	0.8	**3.6**	1.4	**5.0**
727	Flagellar hook protein		CYT	49,168	4.84	0.8	**2.3**	0.9	**3.3**
750	Hypothetical protein		I/II	25,925	9.61	0.9	**1.5**	0.8	**2.1**
767	Translation elongation factor G		CYT	76,824	5.58	0.8	**6.9**	**2.1**	0.8
768	Membrane protein (TmpA)	28	II	37,272	5.08	0.4	**15.3**	**8.2**	**14.2**
769	Outer membrane protein (TmpB)		I	36,947	9.5	1.1	**3.1**	1.3	**2.0**
772	Hypothetical protein		CYT	30,826	8.32	0.9	**3.6**	**1.5**	**5.3**
789	Hypothetical protein		I	29,027	9.71	0.8	1.3	**1.8**	**3.3**
821	Lipoprotein (tpn32)			29,067	7.2	0.8	1.4	1.0	**1.7**
954	Conserved hypothetical protein		II	54,650	9.14	1.1	**1.5**	1.0	**2.1**
956	Hypothetical protein		II	35,991	6.27	1.0	1.3	0.8	**3.5**
971	Membrane antigen, pathogen-specific (TpD)	11	II	22,070	4.56	0.7	**4.1**	**1.6**	**8.2**
974	Hypothetical protein		CYT	10,205	4.57	0.7	1.0	0.9	**1.7**
993	Rare lipoprotein A, putative		I	33,363	9.92	0.7	**1.9**	**2.5**	**5.1**
1015	N utilization substance protein B (pusB)			15,998	9.37	0.9	0.9	0.6	**1.9**
1016	Basic membrane protein (tpn39b)	9	I	39,924	9.16	0.9	**2.1**	**1.5**	**21.5**
1038	Bacterioferrin	20	CYT	19,345	5.33	0.9	1.1	0.8	**2.2**

[a] The chemiluminescence ratio refers to the relative light units resulting from the binding of serum Ig to the *T. pallidum*–GST fusion protein, divided by the value obtained when wells were coated with GST alone. Significant reactivity is indicated by boldface.

[b] Prediction of type I or II signal sequence or N-terminal transmembrane helix (TMH) or cytoplasmic (CYT) location. The precictions were made using the LipoP 1.0 server (12a).

tients. If the development of the humoral immune response between secondary and early latent syphilis in humans coincides with protective immunity, then the 11 proteins that exhibited reactivity only during early latency are of great interest. Four of the 11 proteins, including TP0163 (Mn^{2+}/Mg^{2+} ABC transport, periplasmic binding protein TroA), TP0216 (heat shock protein 70), TP0292 (conserved hypothetical protein), and TP1038 (bacterioferrin), have been previously identified as antigens (3, 17, 20). Five of the seven remaining novel antigens were also identified as antigens in our previous analysis using sera collected from rabbits (13). As in the prior study, it is likely that some false-negative results were obtained due to either a loss of an antigen during the preabsorption of sera with *E. coli* proteins, low expression levels, lability of some of the protein products in *E. coli*, or mutations introduced into ORFs during the cloning process (13). In addition, 131 *T. pallidum* ORFs were not included in this study, either because of the inability to clone the ORF or to convert the ORF to an expressed GST fusion clone or because of mutations in the cloned ORF detected by DNA sequencing (13, 14). Finally, it is possible that the number of antigens detected in the experiments reported here are limited by the number of patient sera used for detection and that further studies with additional sera would detect additional antigens.

The genomic or "reverse vaccinology" approach to defining antigens is a useful method for characterizing the humoral immune response to infectious agents (22). The characterization of antigens such as MglB-2 and TmpC may be useful in immunodiagnosis, in that these antigens give rise to strong, rapid antibody responses that may increase the sensitivity of diagnosis during the early stages of infection. Furthermore, novel vaccine candidates, including potential surface-exposed outer membrane proteins, may be present among the many previously undescribed antigens identified in the human anti-*T. pallidum* immunoproteome. Analysis of the protective capacity of these recombinantly expressed antigens is presently under way in the hope of finding a combination of proteins that protect against *T. pallidum* infection.

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